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Protein Accumulation in Lung Lavage Fluid following Ozone Exposure

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16. ABSTRACT

Accumulation of protein in lung lavage fluid was used as an indicator of pulmonary damage following exposure of guinea pigs to O_3. Exposure of animals to 510, 1000 or 1960 ug/m^3 (0.26, 0.51 or 1.0 ppm) of O_3 for 72 hours resulted in significantly elevated levels of lavage fluid protein when compared to that of air controls. This effect was not observed in animals exposed to 196 ug O_3 (0.10 ppm). When exposure time was reduced to 3 hr, the O_3-induced protein accumulation in lavage fluids was undetectable unless the time of lavage was delayed 10-15 hr following the exposure. Under these conditions, elevated protein content was seen in the lung lavage fluids obtained from animals exposed to O_3 ranging from 510 to 1470 ug O_3/m^3 (0.26-0.75 ppm) and a dose relationship between the amount of protein accumulation in the lung and the concentration of O_3 to which the animals were exposed was observed. Vitamin C deficiency did not enhance this O_3-induced lesion in guinea pigs. The dose relationship has also been confirmed by polyacrylamide gel electrophoresis of the lavage fluids. Lung lavage fluid protein content in animals exposed to 353 ug O_3/m^3 (0.18 ppm) for 8 hr/day for 5 or 10 consecutive days was not different from that of air controls.

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Protein Accumulation in Lung Lavage Fluid following Ozone Exposure[1]

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INTRODUCTION

Accumulation of edema fluid in the lungs is responsible for impaired respiratory function and ultimately mortality in animals exposed to high levels of O_3 (Stokinger, 1965). However, the appearance of gross edema diminishes with decreasing O_3 concentration and with time of exposure, such that it is not detectable by traditional methods (histology or wet weight) in animals exposed to levels of O_3 likely to be encountered in ambient air (Scheel *et al.*, 1959). Recognizing a need to detect subtle changes which might be encountered at lower levels of O_3, Alpert *et al.* (1971) described a method for detecting protein accumulation in the lungs of rats exposed to O_3. ^{131}I-labeled serum albumin was injected intravenously just prior to O_3 exposure, and the amount of ^{131}I recovered in lung lavage fluid at the

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end of exposure was determined. Using this method, they were able to detect an increase in ^{131}I-serum albumin leaking into lungs following exposure to as little as 980 μg O_3/m^3 (0.50 ppm) for 6 hr, whereas histologic evidence of edema was not detectable below 4900 μg/m^3 (2.5 ppm). The accumulation of serum proteins in lavage fluid was taken to be a good indicator of pulmonary toxicity.

In a recent study we used a similar approach to detect subtle changes in guinea pigs exposed to NO_2 (Selgrade *et al.*, 1981). Protein accumulation in lung lavage fluid was detected using the Lowry method (1951). Our data showed that guinea pigs which were vitamin C deficient were much more sensitive to NO_2-induced changes than normal guinea pigs. This observation is relevant to the human population, because guinea pigs, unlike other laboratory rodents, are similar to man in that they require an exogenous supply of vitamin C. Also, there are a number of conditions in the human population which cause individuals to have blood vitamin C levels which are 30–50% lower than normal (Baker *et al.*, 1979; McLeroy and Schendel, 1973; Windsor *et al.*, 1972; Wynn, 1975). In the present paper we have extended this research to determine the effects of O_3 on lung lavage fluid protein content in normal and vitamin C-deficient guinea pigs.

MATERIALS AND METHODS

Animals and diet. Male Hartley guinea pigs (COBS) weighing 300–350 g were obtained from Charles River Farms, Wilmington, Mass., and were used within 6 weeks of arrival. Animals were given food and water *ad libitum*. Normal animals were fed Wayne Guinea Pig Diet (Allied Mills, Inc., Chicago), which is supplemented with 1000 ppm vitamin C. Animals were made vitamin C deficient by feeding on Wayne Rabbit ration (not vitamin C supplemented) for 2–3 weeks prior to and during exposure. This was preferable to feeding commercial preparations specially formulated to produce vitamin C-deficient guinea pigs, because the animals more readily accepted the rabbit chow and the degree of deficiency obtained with rabbit chow was less severe and more representative of the level of deficiency seen in segments of the human population (Selgrade *et al.*, 1981). Due to the variability in different lots of feed, efforts were made to standardize, as much as possible, by milling date of the feed used for these studies, and each animal was tested for blood vitamin C level at the time of sacrifice to determine the extent of deficiency. Blood levels of vitamin C in deficient animals were 25–50% lower than that of normal animals.

O_3 exposures. Animals were exposed to O_3 in individual stainless-steel wire-mesh modules placed in an 11.3-ft^3 Rochester-type chamber (Hinners *et al.*, 1968). Control animals were caged identically and exposed to filtered room air at the same time that O_3 exposures were taking place. O_3 was generated from O_2 using a silent arc discharge O_3 generator (OREC, Phoenix), mixed with filtered room air at a point between the inlet damper and the metering orifice, and introduced into the chamber at a rate of one chamber volume per minute. A mass flow controller was used to control the amount of O_3 entering the chamber. The concentration of O_3 was monitored continuously using chemiluminescent O_3 analyzers (Bendix, Lewisburg, W.V.; *Fed. Reg.*, 1971) which were calibrated biweekly using a Dasibi transfer standard, which was referenced quarterly to a primary ultraviolet O_3 standard (*Fed. Reg.*, 1979; EPA, 1979).

Obtaining lavage fluid. Immediately after exposure or at designated times thereafter, guinea pigs were anesthetized with an intraperitoneal injection of sodium pentobarbital solution (150 mg/animal), blood was obtained in heparinized vacutainers by cardiac puncture for ascorbic acid analysis, and the animals were exsanguinated by severing the abdominal aorta. The trachea was immediately exposed, a slit made just below the larynx, and a catheter inserted and secured with lightweight cord. Ten milliliters of warmed (37°C) saline (0.85%) were then injected into the lungs, allowed to remain for 1 min, withdrawn, and then injected and withdrawn immediately four additional times. Repetitive lavage of the same solution was used, because this resulted in a higher concentration of protein in the lavage fluid. Cells were removed from the lavage fluid by low-speed centrifugation (1000 rpm, 10 min) and were discarded. Fluids were stored at −20°C. Prior to protein analyses, lavage fluids were centrifuged at 27,000*g* (15,000 rpm) for 20 min to remove cellular debris.

Protein analyses. Protein concentrations were determined by the Lowry method (Lowry *et al.*, 1951). Proteins in lung lavage fluids were also analyzed by polyacrylamide gel electrophoresis (PAGE) in 7% discontinuous cylindrical gels (Chrambael and Rodbard, 1971). Approximately 20 μg protein in 100 μl of lavage fluid containing 10% sucrose and a trace amount of bromophenol blue was added to individual tubes with a Hamilton syringe. The gels were electrophoresced for 2 hr at a current of 2.5 mA per gel. Gels were removed from the tubes and stained with coomassie brilliant blue as previously described (Hu *et al.*, 1977), destained in a mixture of methanol, acetic acid, and water (10:3:27 by volume) until the background was cleared, and photographed.

Ascorbic acid analysis. The method described by Roe (1954), with slight modification, was used to determine the amount of ascorbic acid in the blood. Briefly, 0.3 ml blood was added to 1.2 ml cold 5% trichloroacetic acid (TCA), mixed by tapping, and centrifuged for 10 min at 300*g*. Ascorbic acid standard (0.3 ml) was also added to 5% TCA. Then 0.9 ml of the supernatants or ascorbic acid standard was added to 0.3 ml of dinitrophenylhydrazine–thiourea–copper sulfate reagent (2 g:0.25 g:0.03 g in 100 ml of 9 N H_2SO_4), incubated at 37°C for 4 hr, and chilled. Cold 65% H_2SO_4 (1.5 ml) was added to each sample; samples were incubated at room temperature for 30 min, read on a Beckman spectrophotometer (A_{520}), and compared to the standard.

Histologic preparations. Guinea pigs were killed with an intraperitoneal injection of 150 mg per animal of sodium pentobarbital solution. The chest was opened by cutting the sternum along the midline. A small incision was made in the trachea at the larynx, and a glass catheter was inserted and tied with surgical thread. The lungs were filled with fixing solution (0.1 M glutaraldehyde, 0.1 M sodium cacodylate) at a hydrostatic pressure of 20 cm, left for 30 min, removed, and trimmed of any extraneous tissues. Tissues were processed and embedded in paraffin. Sections of 5 μm thickness were made, stained with hematoxylin and eosin, and used for histologic evaluation.

Statistical methodology. The data were analyzed using analysis of variance (ANOVA) and multiple comparison techniques. Factors of interest in the ANOVA were usually vitamin C deficiency and pollutant exposure. If the univariate *F* test for one of these factors or if the test for the interaction of the two

factors were statistically significant, appropriate multiple comparison tests, such as Dunnett's test (Dunnett, 1955) or Duncan's Multiple Range test (Duncan, 1955), were used to determine the pattern of significant effects.

RESULTS

Effect of Ozone Concentration on Lavage Fluid Protein

Normal guinea pigs were exposed for 72 hr to various concentrations of O_3 to obtain the dose–effect relationship of edema formation as measured by protein content of lavage fluid (Fig. 1). Animals exposed to the three highest O_3 concentrations [510 ± 20, 1000 ± 40, or 1960 ± 0 $\mu g/m^3$ (0.26, 0.51, or 1.0 ppm)] had significantly elevated levels of lavage fluid protein compared to animals exposed to air. Animals exposed to 196 ± 35 μg O_3/m^3 (0.10 ppm) had an elevated mean protein concentration compared to that of air controls, but this was not significant. The amount of lavage fluid protein increased rapidly as O_3 concentration increased up to 510 $\mu g/m^3$ (0.26 ppm). Beyond this point there was very little increase in protein with increasing O_3 concentration.

Effect of Lavage Time and Exposure Time

The effects of decreasing the O_3 exposure time to 3 hr and delaying the lavage for various intervals after exposure were examined. Figure 2 shows that the lavage fluid protein content of animals exposed to 1000 ± 40 μg O_3/m^3 (0.51 ppm) for 3 hr and lavaged immediately after the exposure ended was not significantly different from that of air controls. If, however, the time of lavage was delayed 10–15 hr after the end of exposure, a significant increase in lavage fluid protein was observed. By 24 hr after the end of exposure, this effect was no longer detectable.

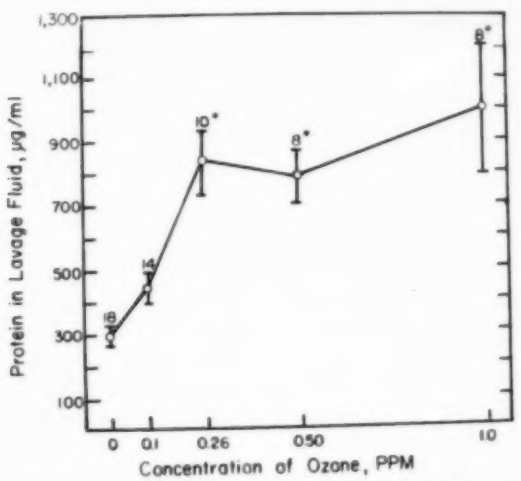

FIG. 1. Dose–response curve for 72-hr exposure to various concentrations of O_3. Points represent means adjusted for experiment obtained from four different experiments that included a total of 6–18 animals per experimental point. Bars indicate standard errors and numbers of animals per experiment are shown above the bars. *Significantly different ($P < 0.05$) from 0 ppm using Dunnett's test.

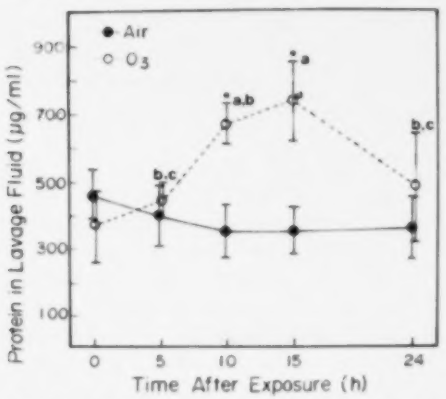

FIG. 2. Effect of delaying time of lavage following exposure of normal guinea pigs to 0.51 ppm O_3 for 3 hr. Each data point represents the average protein of four animals; brackets indicate standard errors. *Significantly different from air controls as determined by Dunnett's test. [a,b,c]For O_3-exposed animals, points sharing the same letter do not differ significantly from one another (Duncan's test).

Consequently, the time of lavage following 3-hr exposures was delayed 15 hr after the exposure for all remaining experiments, except where noted.

Figure 3 shows the effects of varying the exposure time. Normal guinea pigs were exposed to O_3 [510 ± 20 or 1000 ± 40 $\mu g/m^3$ (0.26 or 0.51 ppm)] for 3, 24, or 72 hr. Lavage time was delayed for 15 hr after the 3-hr exposure; however, animals exposed for 24 and 72 hr were lavaged immediately. For all three time periods, the protein content of lavage fluid from animals exposed to either O_3 level

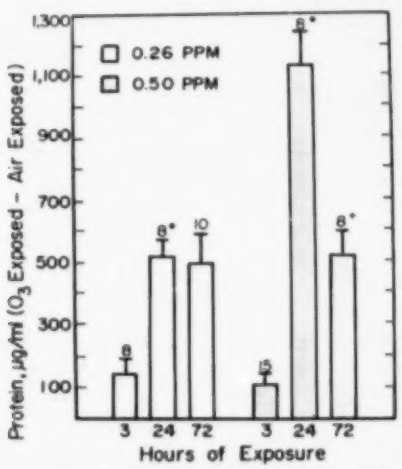

FIG. 3. Effect of various exposure regimens at a given concentration. Bars represent the difference in protein content between O_3-exposed and air controls. Lavage following 3-hr exposures was delayed 15 hr after the exposure ended. For other time periods, animals were lavaged immediately after exposure. *Significant difference ($P < 0.01$) between 3 and 24 hr of exposure for a given concentration. †Significant difference ($P < 0.01$) between 24 and 72 hr of exposure of a given concentration.

was significantly different from that of air controls. At the lower O_3 level, this difference was much larger after 24 hr of exposure compared to 3 hr. At the higher O_3 level, again the effect seen after 24 hr was much greater than that seen after 3 hr and was also greater than that observed after 72 hr.

Effects of 3-hr O_3 Exposures

Figure 4 shows a comparison of the effects of 3-hr exposures to various concentrations of O_3 on lavage fluid protein content of normal and vitamin C-deficient guinea pigs lavaged 15 hr after exposure. When compared to air controls, animals exposed to 196 ± 35 μg O_3/m^3 (0.1 ppm) showed no significant increase in lavage fluid protein concentration regardless of vitamin status; however, animals exposed to higher O_3 concentrations [510 ± 20, 1000 ± 40, and 1470 ± 33 μg/m³ (0.26, 0.51, 0.75 ppm)] did show significant effects. Vitamin C deficiency did not augment this effect. At 1000 μg/m³ (0.5 ppm), vitamin C deficiency alone also caused a slight but significant increase in protein concentration, but ANOVA indicated that there was no interaction between the effect of vitamin C deficiency

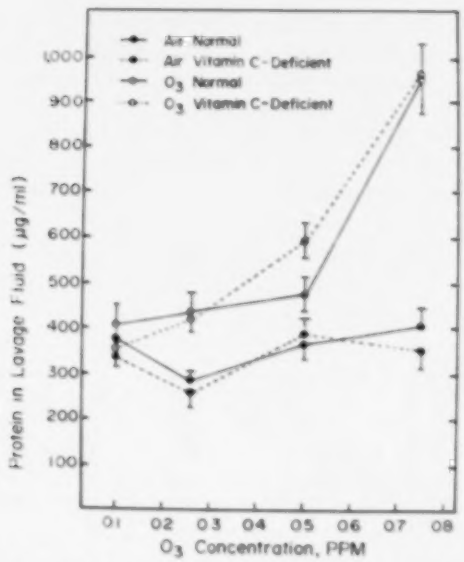

FIG. 4. Effect of vitamin C deficiency and 3-hr exposure to O_3 on protein concentration in lung lavage fluid. Lavage time was delayed 15 hr after exposure. Data points represent means and standard errors from 9–15 animals per group. Results from ANOVA are as follows:

O_3 concentration	Gas		Vit. C def.		Gas × vit. C def.	
(ppm)	*F*	*P*	*F*	*P*	*F*	*P*
0.1	0.38	0.5399	1.42	0.2394	0.15	0.7021
0.26	19.88	0.0001	0.07	0.7999	0.03	0.8638
0.5	28.93	0.0001	7.53	0.0083	2.32	0.1336
0.75	106.2	0.0001	0.03	0.8687	0.87	0.3582

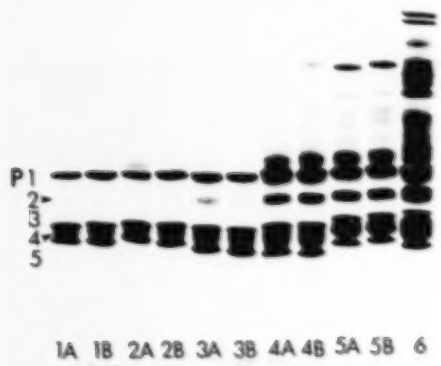

FIG. 5. Polyacrylamide gel electrophoresis of lung lavage fluids following 3-hr exposures to (1) filtered air, (2) 0.1 ppm O_3, (3) 0.26 ppm O_3, (4) 0.51 ppm O_3, and (5) 0.75 ppm O_3, and of normal guinea pig serum (6). A indicates normal animals; B indicates vitamin C-deficient animals.

and O_3. There was no apparent reason for this result, and an effect due to vitamin deficiency alone was not observed at the other three concentrations studied.

Selected lavage fluid samples from the above experiments were subjected to PAGE analysis (Fig. 5). Three major and two weak protein bands were detected in the lavage samples from the normal (1A) and the vitamin C-deficient animal (1B) exposed to filtered air. Exposure to 216 μg O_3/m^3 (0.11 ppm) did not cause any significant change in the electrophoretic profiles of either normal or vitamin C-deficient animals (2A and 2B, respectively); however, significant alterations were seen in animals exposed to higher concentrations of O_3. The intensity of the two weak bands (P2 and P4) is increased in animals exposed to 510 μg O_3/m^3 (0.26 ppm—3A and 3B), and additional bands were observed in samples obtained from animals exposed to 1000 (0.51 ppm—4A and 4B) and 1470 μg O_3/m^3 (0.75 ppm—5A and 5B). The gel profiles suggest a concentration–response relationship. Some of the additional bands appear to be serum proteins as indicated by the presence of matching bands in the serum sample (6). The gel analyses indicate that there is a significant change, both quantitatively and qualitatively, in the protein content in lung lavage fluids obtained from O_3-exposed animals compared to air controls. As with the Lowry protein analyses, there was no evidence on PAGE that this effect was augmented by vitamin C deficiency.

Lung sections from normal and vitamin C-deficient animals exposed to air or O_3 [196, 510, or 1000 $\mu g/m^3$ (0.1, 0.26, or 0.51 ppm)] were also examined for histopathology (two animals per group). No significant lesions were present in normal or vitamin C-deficient animals exposed to air or 196 μg O_3/m^3 (0.10 ppm) for 3 hr. A minimal to slight bronchiolitis was observed in terminal bronchioles of both normal and vitamin C-deficient animals exposed to 510 μg O_3/m^3 (0.26 ppm), while animals exposed to 1000 μg O_3/m^3 (0.51 ppm) for 3 hr showed a similar bronchiolitis and also minimal to slight alveolitis. Figure 6 shows representative histological sections for normal and vitamin C-deficient animals exposed to air or 1000 μg O_3/m^3 (0.51 ppm) for 3 hr.

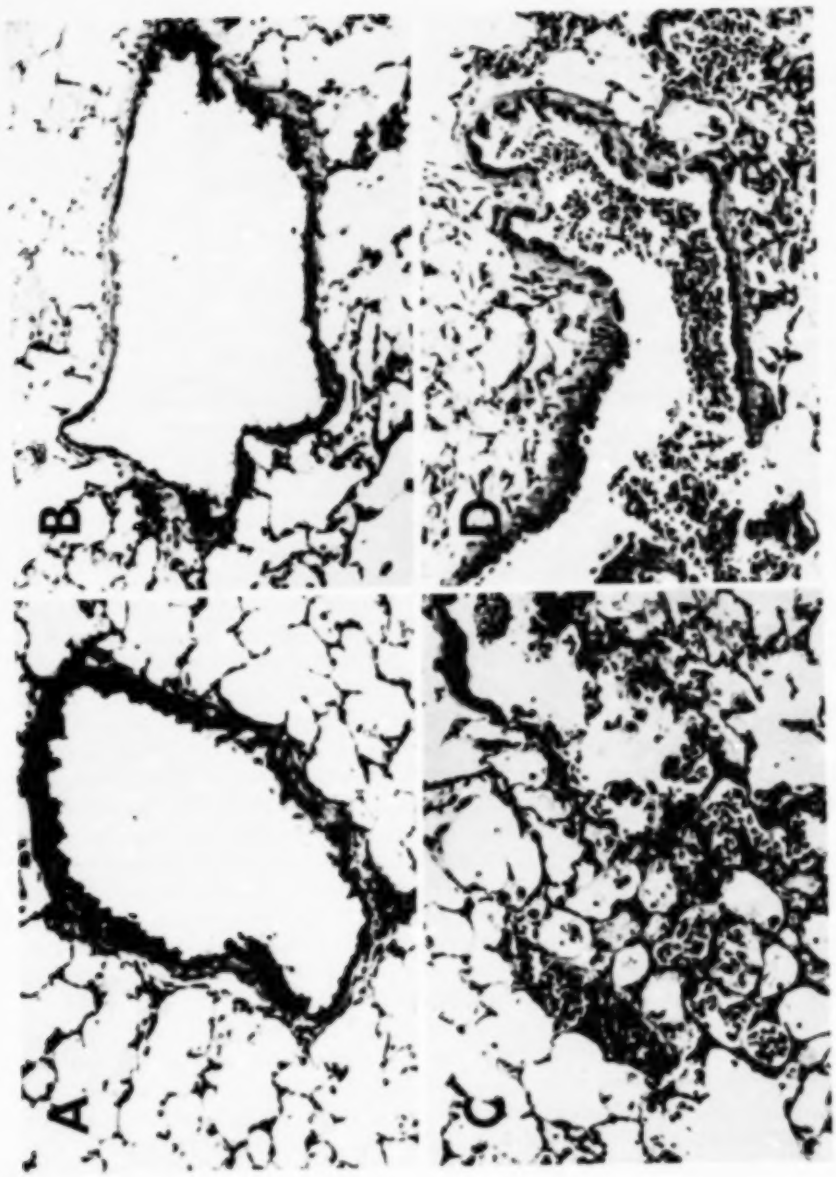
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TABLE 1
EFFECT OF EXPOSURE TO 0.18 ppm O_3 ON LUNG LAVAGE FLUID PROTEIN CONCENTRATION

	3 hr[a]	8 hr × 5 days	8 hr × 10 days
Air	417 ± 29 (18)[b]	487 ± 48 (12)	418 ± 35 (12)
O_3	470 ± 29 (18)	429 ± 31 (12)	470 ± 33 (12)

[a] Lavage time was delayed 15 hr after exposure.
[b] Mean protein concentration in μg/ml ± standard error (no. of animals).

Low-Level Acute and Chronic Exposures

Finally, normal guinea pigs were exposed to 353 ± 16 μg O_3/m^3 (0.18 ppm) for a single 3-hr exposure or for 8 hr/day for 5 or 10 consecutive days. Table 1 shows that none of these exposure regimens produced significant differences in lavage fluid protein content when compared to air controls.

DISCUSSION

The present study investigated protein accumulation in lung lavage fluid of guinea pigs as an indicator of pulmonary damage following O_3 inhalation. O_3-exposed animals showed significant increases in lavage fluid protein compared to air controls at concentrations ranging from 510 to 1960 μg O_3/m^3 (0.26–1.0 ppm) and exposure times ranging from 3 to 72 hr. Regardless of the exposure time, the lowest concentration of O_3 at which effects were observed was 510 μg/m³ (0.26 ppm). Animals exposed to 353 μg O_3/m^3 (0.18 ppm) for 3 hr were no different from air controls, nor were animals exposed to this same concentration 8 hr/day for 5 or 10 days. In this study, as in others (Chow *et al.*, 1974; Dungworth *et al.*, 1975; Stephens *et al.*, 1974), the amount of damage due to O_3 was much more dependent on concentration than time of exposure, since 196 μg O_3/m^3 (0.10 ppm) produced no effects even after 72 hr of continuous exposure, whereas 510 μg O_3/m^3 (0.26 ppm) produced effects after as little as 3 hr. A comparison of vitamin C-deficient and normal animals showed no difference in their susceptibility to O_3-induced protein accumulation.

Normal animals exposed to O_3 for 3 hr did not show significant increases in lavage fluid protein content unless the time of lavage was delayed 10–15 hr after the exposure ended (Fig. 2). This type of delayed effect has also been observed with the histological appearance of edema following O_3 exposure (Scheel *et al.*, 1959) and is very similar to the delayed effect seen following exposure of vitamin C-deficient animals to NO_2 (Selgrade *et al.*, 1981).

Animals exposed to 510 or 1000 μg O_3/m^3 (0.26 or 0.5 ppm) for 24 hr and lavaged immediately showed a much greater increase in lavage fluid protein compared to air controls than animals exposed to the same concentration for only 3 hr with

FIG. 6. Lung tissues from (A) normal animal exposed to air, (B) vitamin C-deficient animal exposed to air, (C) normal animal exposed to 0.51 ppm O_3 for 3 hr, and (D) vitamin C-deficient animal exposed to 0.51 ppm O_3 for 3 hr. Mild alveolitis can be seen in normal and vitamin C-deficient animals exposed to O_3 (C) and (D). Occasionally bronchiolitis can be seen in vitamin C-deficient animals exposed to O_3 (D). (Original magnification 330×, hematoxylin and eosin stain)

lavage delayed. At 510 μg O_3/m^3 (0.26 ppm) there was no difference in the effects seen after 24 or 72 hr of exposure, and at 1000 μg O_3/m^3 (0.51 ppm) there was actually less effect seen at 72 than at 24 hr. This correlates with the morphologic studies of Stephens *et al.* (1974), who observed histologic evidence of repair which was most obvious between 24 and 48 hr after onset of O_3 exposure. During this "reparative–adaptive period" new cells resulting from a marked increase in proliferation of type II cells appeared to be resistant to O_3, whereas many of the original cells were injured or destroyed. Also, there are many other examples in the literature of O_3-induced tolerance which could be related to our result (Stokinger, 1957; Stokinger and Scheel, 1962; Fairchild, 1967; Jaffe, 1967; Gardner *et al.*, 1972).

Vitamin C-deficient animals exposed for 3 hr to concentrations of O_3 ranging from 196 to 1470 $\mu g/m^3$ (0.10–0.75 ppm) were no more sensitive to O_3-induced protein accumulation than their normal counterparts. This result was somewhat surprising since there are several reports which show that exogenous ascorbic acid protects mice against O_3-induced edema and mortality (Matzen, 1957; Mittler, 1958; Pagnotto and Epstein, 1969). Also, collagen synthesis is enhanced in O_3-exposed animals presumably in response to tissue destruction (Hassdin *et al.*, 1976). Such a repair mechanism might be lost in vitamin C-deficient animals, since vitamin C plays an important role in collagen production, as well as vascular permeability (Barnes, 1975; Moran and Green, 1979). In contrast to the present study, vitamin C-deficient animals were much more sensitive to NO_2-induced protein accumulation than normal animals (Selgrade *et al.*, 1981). The fact that vitamin C deficiency greatly enhances NO_2-induced protein accumulation but does not effect O_3-induced protein accumulation suggests that the mechanisms of action and/or the mode of detoxification of these two pollutants differ. Chow *et al.* (1974) suggested that the mechanism of action of these two oxidants is different, based on differences he observed in lung glutathione peroxidase levels and serum lysozyme levels following exposure. Also, studies on the effects of NO_2 and O_3 on pentobarbital-induced sleeping time suggest that these two pollutants produce extrapulmonary effects by different means (Miller *et al.*, 1980; Graham *et al.*, 1981). It appears from our studies that ascorbic acid has a greater role in detoxifying NO_2 than O_3. Because of its greater water solubility, NO_2 may more effectively oxidize ascorbic acid in lung tissue. We have found that aqueous solutions of ascorbic acid are more readily oxidized in atmospheres of NO_2 than in atmospheres of O_3 (unpublished observations).

The data presented here illustrate the usefulness of Lowry protein determinations on lung lavage fluid as a rapid quantitative means for assessing subtle edematogenic changes in animals exposed to O_3. Although some of the additional protein may be the result of increased cellular catabolism during O_3 exposure (Dillard *et al.*, 1972), the main source is probably serum protein. This is suggested by the appearance of extra protein bands on PAGE which co-migrate with serum proteins and by Alpert *et al.*'s (1971) work in which ^{131}I-labeled serum albumin injected in the blood stream was recovered in lavage fluid. The histopathologic changes observed following exposure to 510 and 1000 μg O_3/m^3 (0.26 and 0.51 ppm) are those associated with edema. No histopathologic lesions were observed

in animals exposed to 196 μg O_3/m^3 (0.1 ppm), which correlated with the lack of effect on protein accumulation. Using this protein determination method, edematous changes could be demonstrated following exposure to as little as 510 μg O_3/m^3 (0.26 ppm) for 3 hr.

A comparison of results from the present O_3 study with those from our previous NO_2 study (Selgrade *et al.*, 1981) suggests that O_3 is more toxic than NO_2. Following a 72-hr exposure edema was observed at 510 μg O_3/m^3 (0.26 ppm) but not at 9400 μg NO_2/m^3 (5 ppm). Vitamin C deficiency appears to greatly narrow this difference in toxicity, since animals exposed to 1960 μg O_3/m^3 (1.0 ppm) for 72 hr had lavage fluid protein content similar to that seen in vitamin C-deficient animals exposed to 1880–5640 μg NO_2/m^3 (1–3 ppm) for 72 hr. While vitamin C-deficient animals appear to be at greater risk of NO_2 toxicity, this deficiency does not seem to enhance the toxic effects of O_3.

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